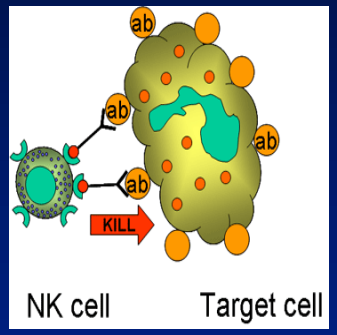
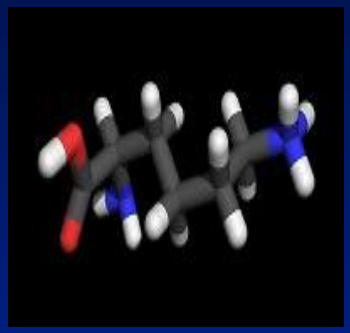
Novel anti-infective molecule from innate immune cells as an antibiotic-alternative to control infections caused by Apicomplexa



Hyun S. Lillehoj

Animal Parasite Disease Laboratory
Animal & Natural Resources Institute
United States Department of Agriculture
Agricultural Research Service
Beltsville, MD, USA



International Symposium "Alternatives to Antibiotics: Challenges and Solutions in Animal Production"
OIE, Paris, September 25-28, 2012

Contributing scientists and collaborators

Poultry Mucosal Pathogen Research Team
Beltsville Agricultural Research Center

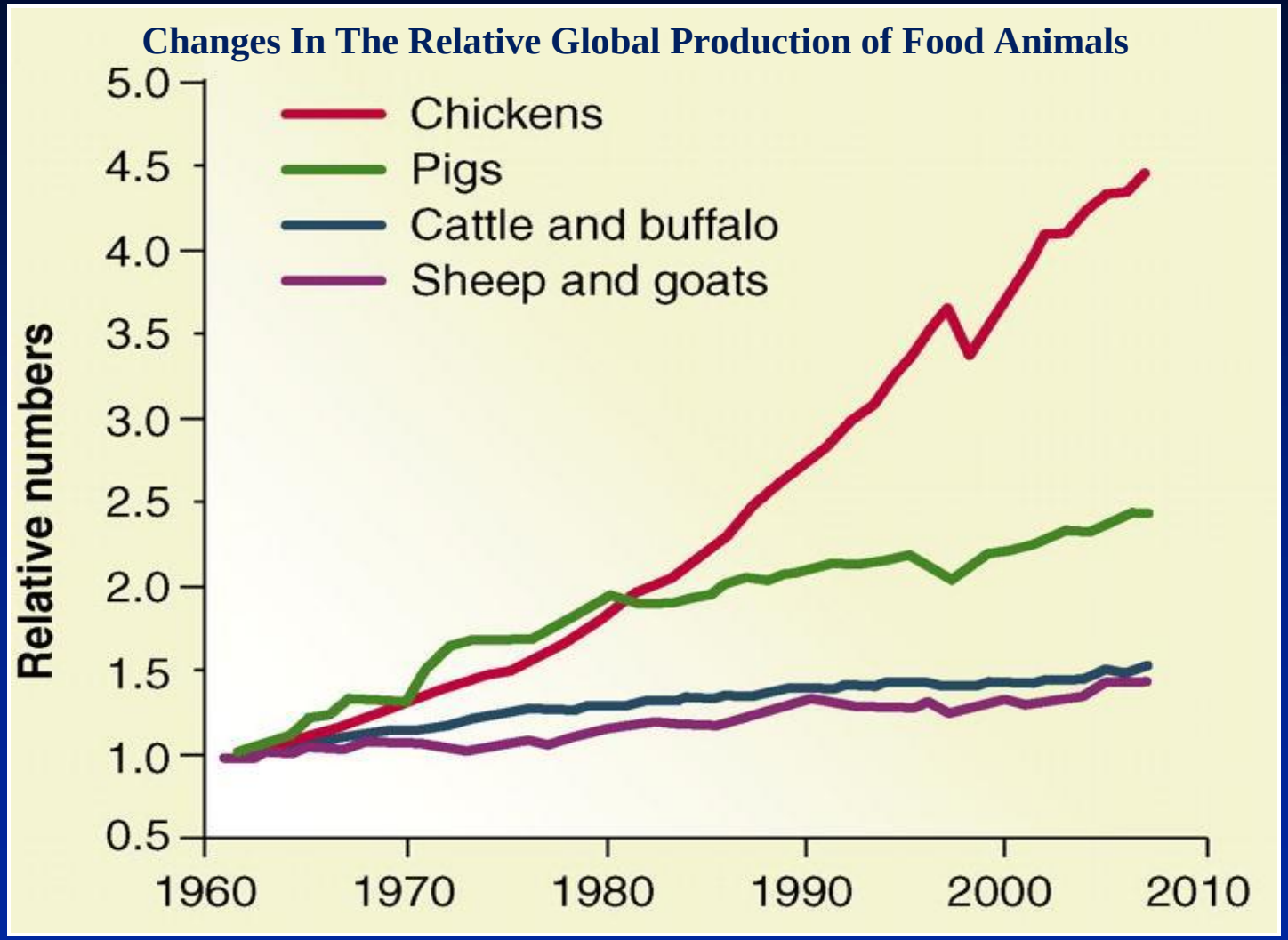
Chung-Ang University, South Korea

Yeong Ho Hong
Geun Bae Kim

Global food security/food safety:

Poultry production is projected to rise the most among the meats over the next decade as poultry is the most efficient feed-to-meat converter.

USDA Long-term Projections, February, 2010



Godfray et al., Science 327:812-818, 2010.

Current Challenges in Poultry Production: Just few examples….

Antibiotics/AGPs Organic farming

Climate changes

Biofuels/Bioproducts

Biosecurity

Animal well-being

Food safety

Global food security

Coccidiosis

Necrotic enteritis

Gangrenous dermatitis

Dysbacteriosis

Campylobacter

Gut microbiota

Nutrition/diet Innate immunity

Industry: Safe food, efficient, cost-effective, affordable, quality of food, nutrient profiling, waste management, science-based technology…..

COMMENT

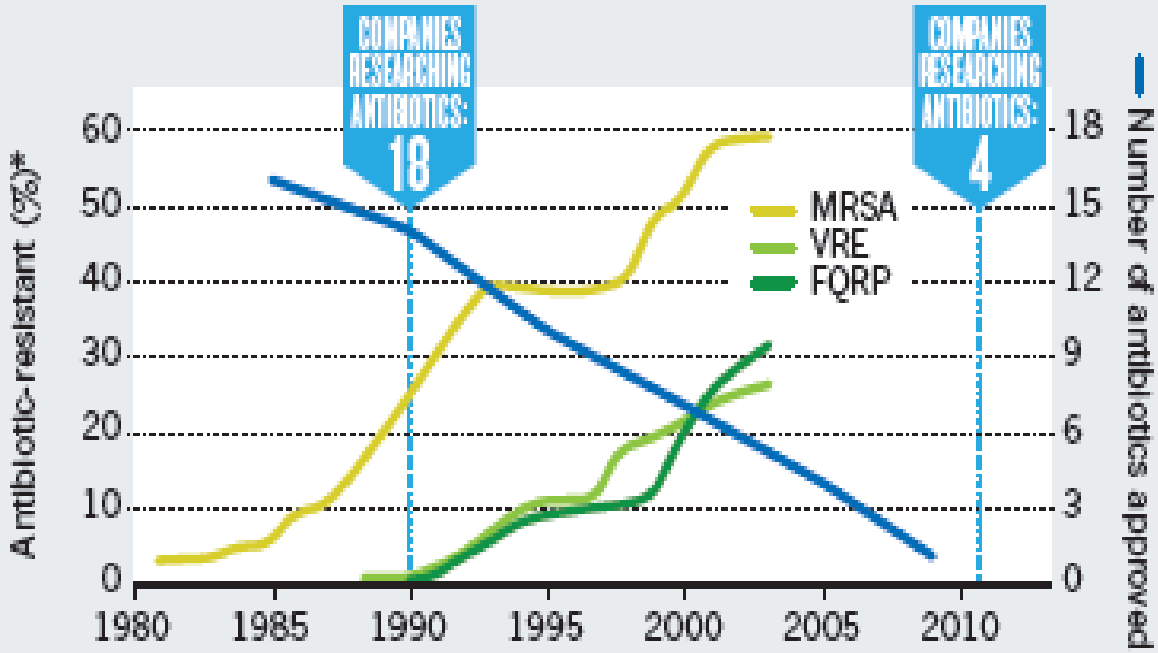
Fix the antibiotics pipeline

As resistance mushrooms, governments must make development of new antibiotics financially viable for industry, say **Matthew A. Cooper** and **David Shlaes**.

COMMENT 32 | NATURE | VOL 472 | 7 APRIL 2011

A PERFECT STORM

As bacterial infections grow more resistant to antibiotics, companies are pulling out of antibiotics research and fewer new antibiotics are being approved.



*Proportion of clinical isolates that are resistant to antibiotic. MRSA, methicillin-resistant *Staphylococcus aureus*. VRE, vancomycin-resistant *Enterococcus*. FQRP, fluoroquinolone-resistant *Pseudomonas aeruginosa*.

“Get pigs off antibiotics”

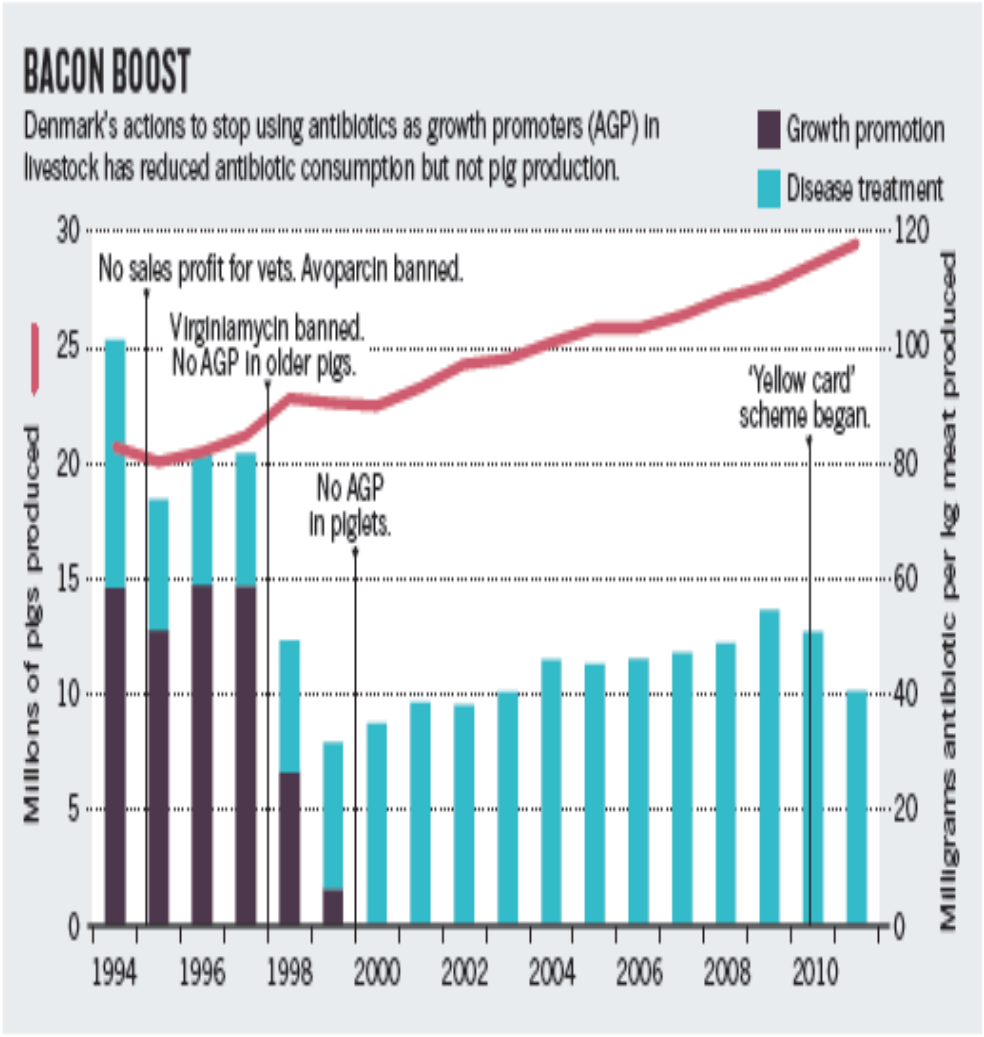
Frank Aarestrup explains how he helped Denmark to cut the use of antibiotics in its livestock by 60%, and calls on the rest of the world to follow suit

T. STODDART/GETTY

Pork production has risen steadily in Denmark, despite a voluntary halt to using antibiotics to boost growth.

Get pigs off antibiotics

Frank Aarestrup explains how he helped Denmark to cut the use of antibiotics ir its livestock by 60%, and calls on the rest of the world to follow suit.



Anticoccidials: EU and Asia regulations

EUROPEAN COMMISSION TOGETHER SINCE 1957

Directorate-General for Health & Consumers

European Union Register of Feed Additives
pursuant to Regulation (EC) No 1831/2003

Appendix 3a. List of modifications to the Register, versions 1-50

(Status: Released 12 November 2010.)

Directorate D - Animal Health and Welfare
Unit D2 – Feed

Additive	Commercial name	Date authorization/ Entry in register	Expiry date
Decoquinate, 60.6 g/kg	Deccox	03.2007	03.2017
Monensin sodium	Elancoban G100, G200	07.2004	07.2014
	Coxidin	02.2007	02.2017
Robenidine hydrochloride 66 g/kg	Cycostat 66 G	12.2005	10.2014
Lasalocid A sodium 15g/100g	Avatec 150G	12.2005	08.2014
Narasin 100g/kg	Monteban G100	04.2006	08.2014
Salinomycin sodium 120g/kg	Sacox 120 microGranulate	05.2007	08.2014
	Salinomax 120G	05.2007	04.2015
	Kokcisan 120G	02.2008	02.2018
Maduramicin ammonium alpha 1g/100g	Cygro 1%	11.2005	Application submitted
Diclazuril 0.5g/100g	Clinacox 0.5%	11.2005	Application submitted
Narasin 80g/kg- Nicarbazin 80 g/kg	Maxiban G160	10.2010	10.2020
Semduramicyn sodium	Aviax 5%	10.2010	10.2020
Nicarbazin 250 g/kg	Nicarbazin	10.2010	10.2020

http://ec.europa.eu/food/food/animalnutrition/feedadditives/registeradditives_en.htm

News in brief

Korea to ban antibiotics in livestock feed

South Korea will ban the use of antibiotics in feed for livestock, poultry and

Chile aims to ban antibiotics in aquaculture

06 Jun 2008

An international campaign aimed at forbidding the use of antibiotics in aquaculture was launched yesterday in Chile. The project also demands that all sanitary standards regarding antibiotics for Chilean salmon consumers be brought in line with standards such as the United States' FDA rules or those of the European Union.

ENTERIC HEALTH

Diseases

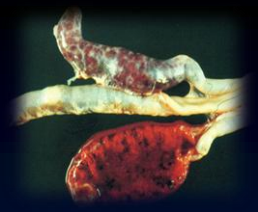
Coccidiosis

>$ 3.2 billion:

Necrotic Enteritis

>$ 2 billion

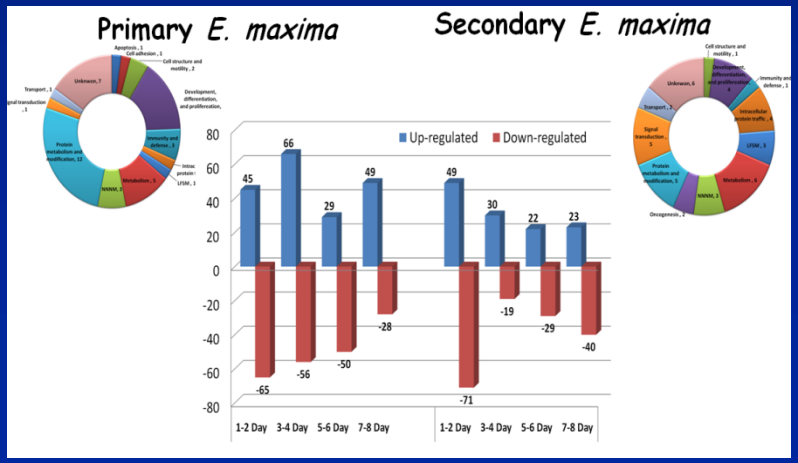
Coccidiosis and Necrotic Enteritis



- In the United States, necrotic enteritis (**NE) and** coccidiosis are among the most important infectious diseases in chickens (Smith and Helm, 2008). Clinical signs of coccidiosis include sudden loss of body weight, diarrhea, gross lesion, hemorrhage.
- Domestic chickens are host to several distinct species of *Eimeria*. Field infections are mixed. Infection with *Eimeria* spp. leads to protection against homologous but not against heterologous spp. Coccidiosis is a primary risk factor for NE.
- The etiologic agent of NE is *Clostridium perfringens, a gram-positive, anaerobic, spore-forming* bacterium that is transmitted by the fecal-oral route as well as through contaminated feed, water, housing structures, and insects (Williams et al., 2003).
- Increasing trends of legislative restrictions of AGPs worldwide has contributed to enhanced coccidiosis and NE outbreak.

Species-specific protective immunity: a challenge to develop a multivalent coccidiosis vaccine

	E. acervulina	E. maxima	E. tenella
Average L x W	18.3 x 14.6	30.5 x 20.7	22.0 x 19.0
Tissue location	Epithelial	Gametocytes Subepithelial	2nd generation schizonts Subepithelial
Prepatent period	97 hrs	121 hrs	115 hrs
Sporulation time	17 rs	30 hrs	18 hrs

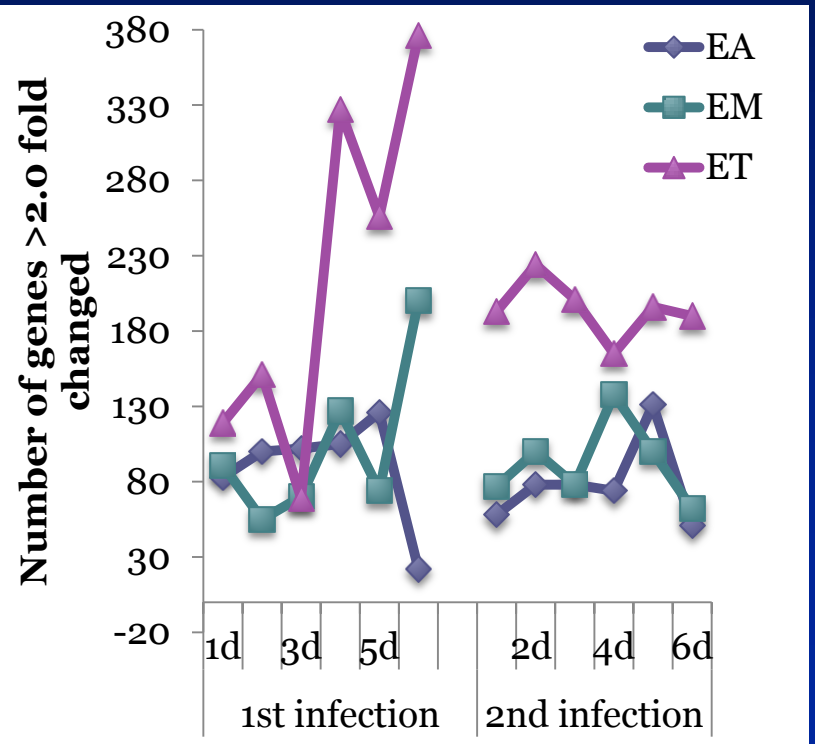


Kim et al. 2011. PlosOne V6:e27712

PLoS one

Comparative Microarray Analysis of Intestinal Lymphocytes following *Eimeria acervulina*, *E. maxima*, or *E. tenella* Infection in the Chicken

Duk Kyung Kim[1], Hyun Lillehoj[1]*, Wongi Min[2], Chul Hong Kim[1], Myeong Seon Park[1], Yeong Ho Hong[3], Erik P. Lillehoj[4]



Alternatives to AGP?

How do they work? Safety? Synergy? Stability? Interrupt microbiota? Work on my chicken strain? Virus? Bacteria? Parasites?

AMPs

Herbs/ essential oils

Enzymes

Bacteriophages

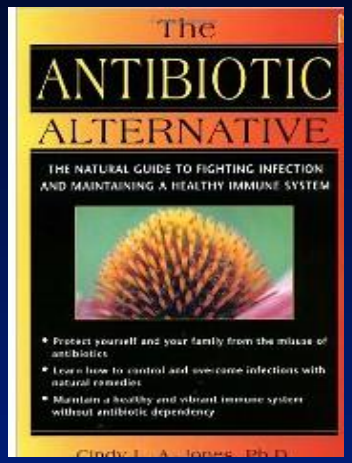
Recombinant vaccines

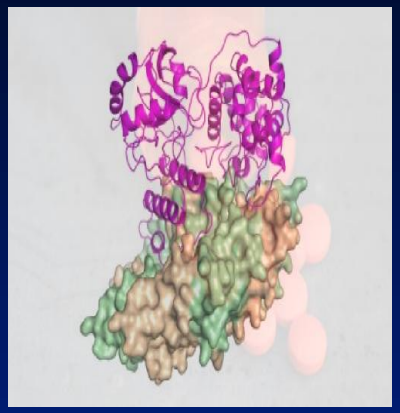
TLR agonists

Prebiotics/ probiotics

Hyperimmune antibodies

Antibiotics alternatives for coccidiosis?

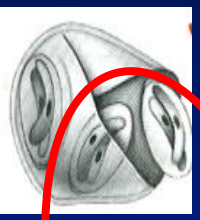


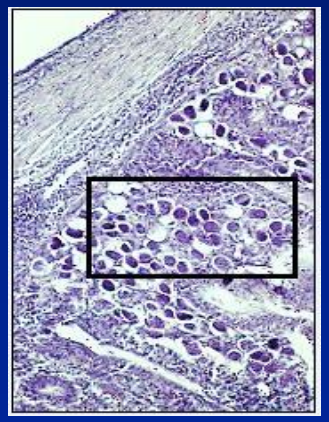


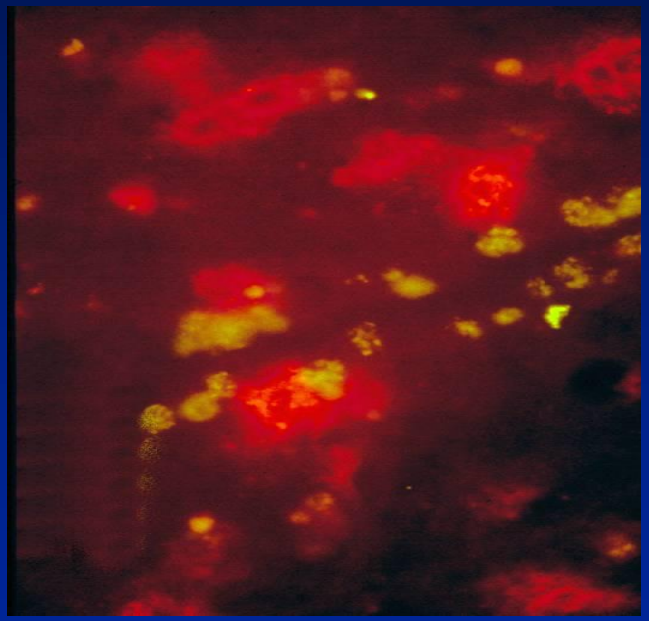
Identify innate immune molecules that:

1) have broad spectrum anti-coccidial activities,
2) do not elicit host resistance against them,
3) are not labor-intensive for applying for industry

Initial interaction of sporozoites with macrophages starts local inflammatory response (innate) which triggers cell infiltration (Innate immune cells) and long-lasting memory (adaptive) immune response







CD8

Eimeria

Sporozoites in macrophages
2-4 hrs after invasion

Local inflammatory response, lymphocyte infiltration, cytokine production (Th1, Th2, Th17, Treg)

Lillehoj , 2006. Exp. Rev. Vaccines 5 (1):143-163.

Importance of IEL NK cells in resolution of intracellular parasitism in poultry coccidiosis

Table 5. Comparative NK-cell activity of IEL from the duodenum, jejunum, ileum and caecum with that of spleen cells

		% cytotoxicity				
Target cell	E:T*	Duodenum	Jejunum	Ileum	Caecum	Spleen
RP9	100:1	12·0	17·3	30·2	−3·7	39·6
	50:1	7·3	16·0	12·1	0·5	17·3
MSB-1	100:1	9·7	26·3	28·0	9·8	9·7
	50:1	14·8	27·6	33·0	13·9	14·8
RP12	100:1	2·6	ND	ND	0·1	1·3
	50:1	−2·3	−6·2	ND	ND	−2·3
CU36	100:1	11·4	35·1	39·4	26·8	11·4
	50:1	17·8	34·7	42·9	23·5	17·8

Values are mean of triplicate wells in a 12-hr ^{51}Cr-release assay and standard deviations were usually less than 20% of the mean.
ND, not determied
* Effector cells were obtained from SC chickens.

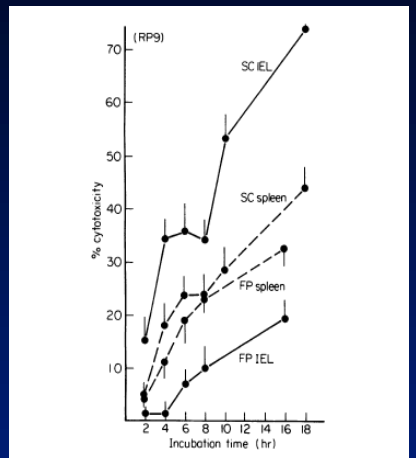


Figure 1. Kinetics of NK-cell activity of IEL and spleen cells obtained from SC and FP chickens. Target cells are RP9. Values represent the mean cytotoxicity with standard deviation of triplicate wells at E:T ratio of 50:1.

Chicken IELs are composed of 21% CD3+, 70% CD8+, and 9% CD4+ T lymphocytes with about 30% of the CD8+ subset possessing NK cell activity

Lillehoj, HS ans Chai, JY. 1988. Dev. Comp. Immunol. 12(3):629-643.

Chai, JY, and Lillehoj, HS. 1988. Immunology 63(1):111-117.

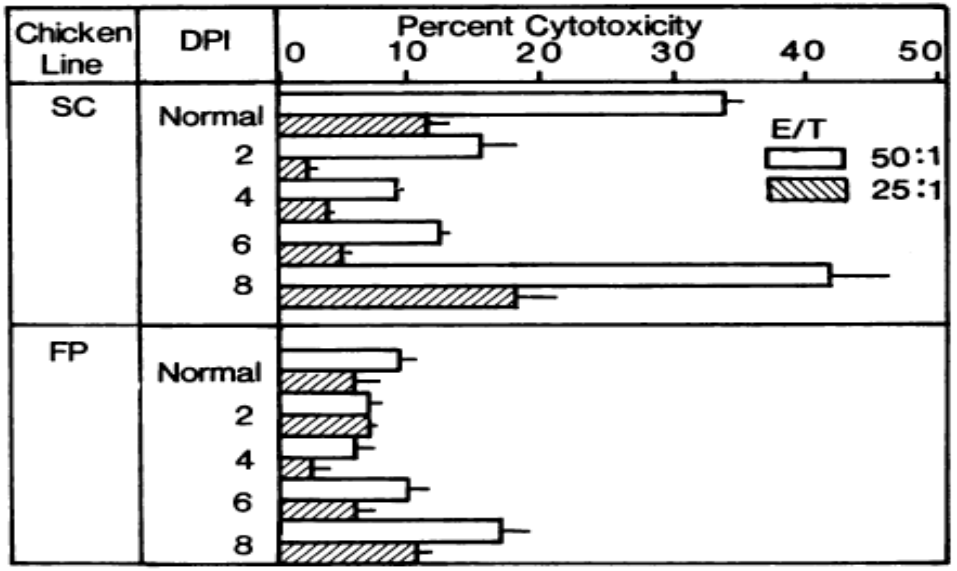
INFECTION AND IMMUNITY, July 1989, p. 1879–1884
0019-9567/89/071879-06$02.00/0

Vol. 57, No. 7

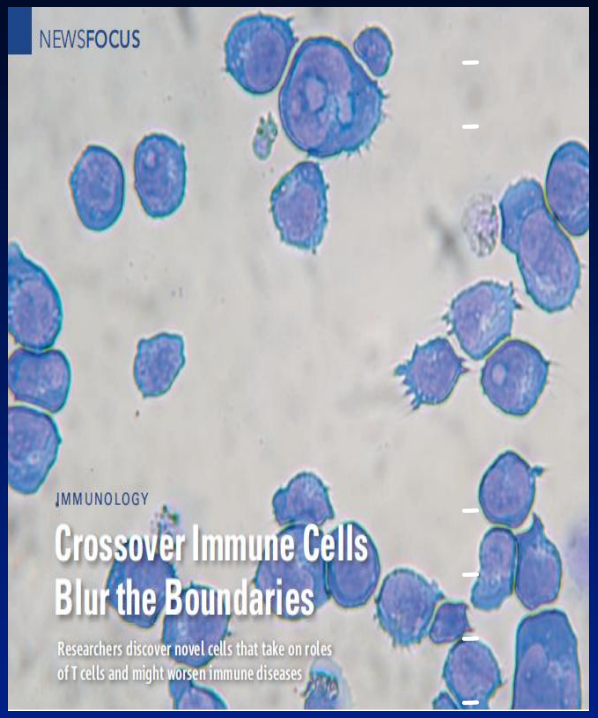
Intestinal Intraepithelial and Splenic Natural Killer Cell Responses to Eimerian Infections in Inbred Chickens

HYUN S. LILLEHOJ

Protozoan Diseases Laboratory, Livestock and Poultry Sciences Institute, Agricultural Research Service, BARC-E, Building 1040, U.S. Department of Agriculture, Beltsville, Maryland 20705



In human: An antimicrobial activity of cytolytic T Cells are mediated by granulysin.



CTLs kill intracellular pathogens by a granule-dependent mechanism.

Granulysin, a protein found in granules of CTLs, reduce the viability of a broad spectrum of pathogenic bacteria, fungi, and parasites in vitr

Granulysin directly kills *Mycobacterium tuberculosis* altering the membrane integrity of the bacillus in combination with perforin, decreased the viability of intracellular *M. tuberculosis*. (Linde et al., 2005. Infect. Immun. 73:6332).

In chickens, no appropriate surface markers efficiently discriminate chicken NK cells from T lymphocytes. CD4+ and CD8+ cells expressed equal amounts of NK lysin, but IELs depleted of CD8+ cells exhibited reduced NK-lysin transcript levels.

Hong et all, 2008, Avian Diseases 52: 302-305.

Discovery of avian homologue of NK lysin involved in local response to *Eimeria* using genomics

Sampling ➡ hybridization ➡ analysis ➡ normalization ➡ bioinformatics ➡ target genes

Clusters That Contain More Than 14 ESTs

Contig ID	Gene description	Organism	Accession No.	No. of ESTs
Contig171	NK-lysin	Equus caballus	CD728315	87
Contig1648	Apolipoprotein AIV	Gallus gallus	CD731936	69
Contig42	Fatty acid binding protein	Gallus gallus	CD735219	51
Contig1279	Immunoglobulin α heavy chain	Gallus gallus	CD735924	43
Contig1234	2',5'-oligoadenylate synthetase	Gallus gallus	CD730844	24
Contig944	ATP synthase β-subunit	Cyprinus carpio	CD732620	24
Contig971	Interferon regulatory factor 6	Ovis aries	CD732407	22
Contig1300	Jun-binding protein	Gallus gallus	CD739778	20
Contig1325	Acidic ribosomal phosphoprotein (P0)	Gallus gallus	CD737516	20
Contig1000	Angiotensin converting enzyme	Gallus gallus	CD731489	19
Contig608	α-tubulin	Gallus gallus	CD736033	19
Contig1524	34/67 kDa laminin receptor	Cricetulus griseus	CD737204	18
Contig1792	Actin related protein 2/3 complex, subunit 1B (ARPC1B)	Homo sapiens	CD737537	18
Contig733	GAPDH	Gallus gallus	CD735039	18
Contig992	Unknown	Unknown	CD729072	18
Contig528	[illegible]	[illegible]	[illegible]	17
Contig352	Unknown	Unknown	CD733292	16
Contig1247	Ferritin heavy chain	Gallus gallus	CD740150	15

Min et al., 2005. Molecular Biotechnology 30:143-150

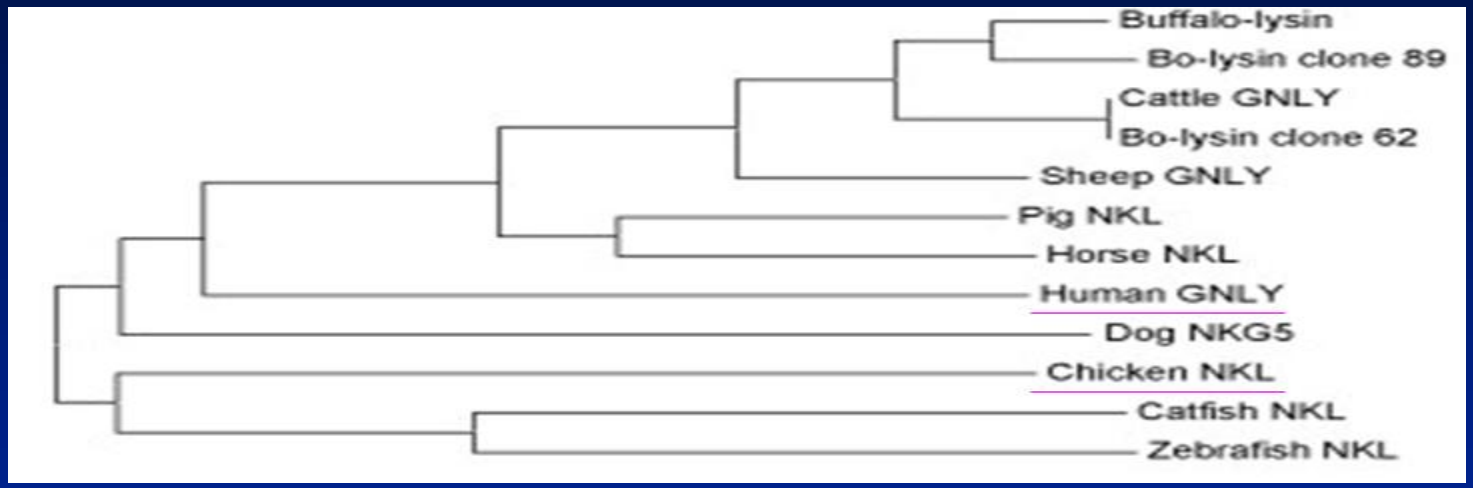
```
                                                1   2                                3
NK-lysin, chicken      ---------------MAAALIVLLALGAAVQVAVTEPPRDDHRDLDAGSHWEQQWHLLQD   45
NK-lysin, porcine      -------------------------------PGLAFSGLTPEHSALARAHPCDGEQFCQN   29
NK-lysin, bovine       ------------------------------------------------------------
NK-lysin, equine       MKKMGCGGRLSSCPTMTSRALLLLASALLGTPGLTFSGLNPESYDLATAHLSDGEQFCQG   60
granulysin, human      ---------------MATWALLLLAAMLLGNPGLVFSRLSPEYYDLARAHLRDEEKSCPC   45

                                                1   2                                3
NK-lysin, chicken      GSAAWDADEGDAMGPGKGIKCRFCVSLVKKVQKIVGDDPDEDAINNALNKVCSTGR-RQR   104
NK-lysin, porcine      LAPEDPQGDQLLQREELGLICESCRKIIQKLEDMVGPQPNEDTVTQAASRVCDKMK-ILR   88
NK-lysin, bovine       ---EDPQGDLLLQGEELSLRCGSCRRIIQHLMDKLGDQPDENTVIEEASKVCSKMR-LLK   56
NK-lysin, equine       LTQEDLQGDLLTERERQGIACWSCRKILQKLEDLVGEQPNEATINEAASRVCRNLG-LLR   119
granulysin, human      LAQEGPQGDLLTKTQELGRDYRTCLTIVQKLKKMV-DKPTQRSVSNAATRVCRTGRSRWR   104
                              .:         .     *  ::::: . :  .* : :: :  .:** .     :

                         4
NK-lysin, chicken      SICK----------------------------------------QLLKKLRQQLSDALQNN   125
NK-lysin, porcine      GVCK----------------------------------------KIMRTFLRRISKDILTG   109
NK-lysin, bovine       GLCKSIMKKFLRTIAEDIVAGKTSQVICVDIKMCKSKPVGFIKKIMRTCLRLISRDILAG   116
NK-lysin, equine       GACK----------------------------------------KIMRTCLRLISRDILAG   140
granulysin, human      DVCR----------------------------------------NFMRRYQSRVTQGLVAG   125
                       . *:                                        ::::     ::  :  .

                            5     6
NK-lysin, chicken      DDPRDVCTTLGLCKG-----   140
NK-lysin, porcine      KKPQAICVDIKICKEKTGLI   129
NK-lysin, bovine       KKPQEVCVDIKLCKHKAGLI   136
NK-lysin, equine       KKPQEVCVDIKLCKHKAGLI   160
granulysin, human      ETAQQICEDLRLCIPSTGPL   145
                       . .: :*  : :*
```

Comparison of predicted amino acid sequences of chicken, porcine, bovine and equine NK-lysin and human granulysin using the CLUSTAL W (1.82) program. The six conserved **cysteine residues** are numbered 1-6. Asterisks (*) indicate identical amino acid residues.

Hong et all, 2006, Molecular cloning and characterization of chicken NK-lysin. Vet. Immunol. Immunopathol. 110:339-47.

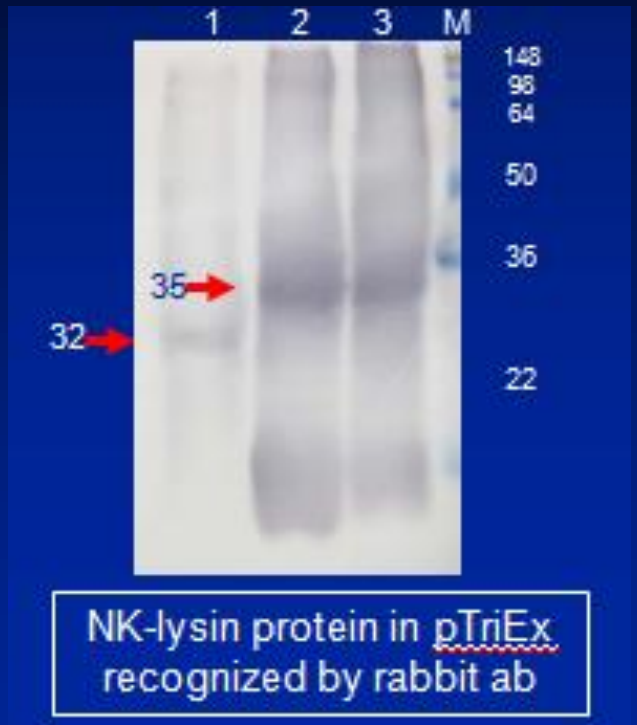
Phylogram based on deduced amino acids sequences of buffalo and various species GNLY/NK-lysin.

A **phylogenetic tree** or **evolutionary tree** is a branching diagram or "tree" showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancester.

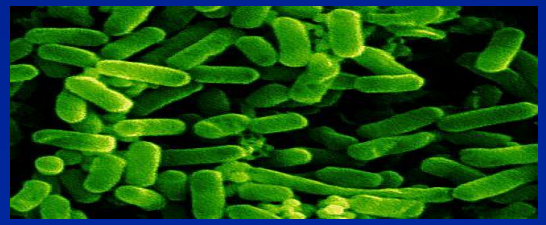


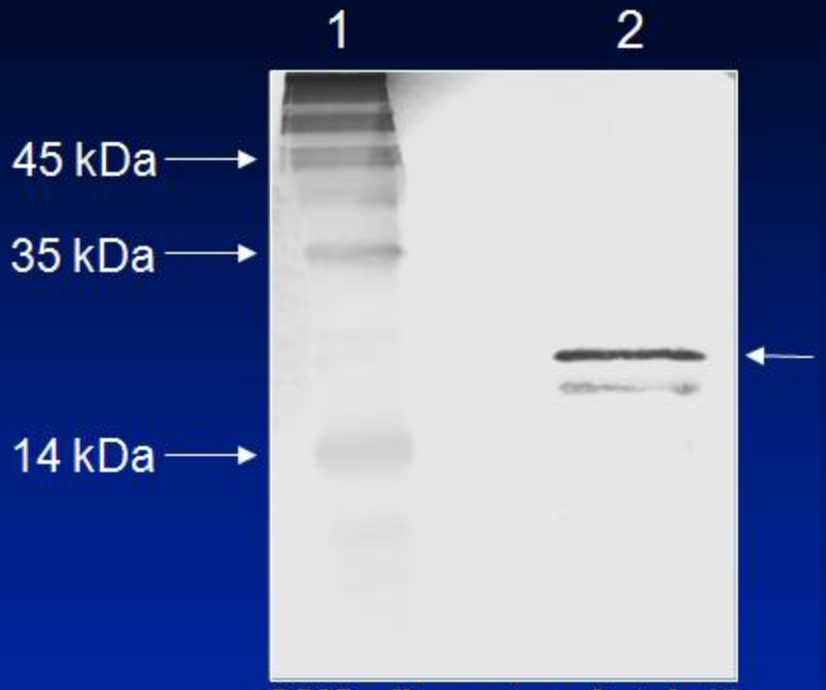
- Comparison of these amino acid sequences using the CLUSTAL W (1.82) program revealed 15% identity of chicken NK-lysin to the porcine and human proteins, 14% to bovine NK-lysin, 17% to equine NK-lysin.
- Comparison of mammalian NK-lysins and human granulysin among themselves revealed levels of homology ranging 36% (bovine NK-lysin versus human granulysin) and 67% (porcine versus equine NK-lysin).

Biochemical characterization and recombinant chicken NK lysin expression

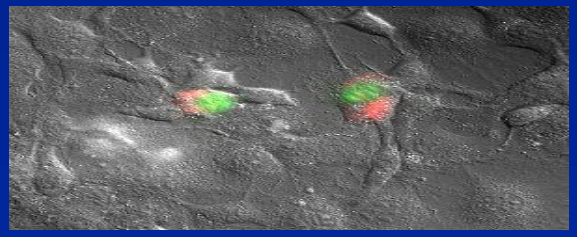


NK-lysin protein in pTriEx recognized by rabbit ab

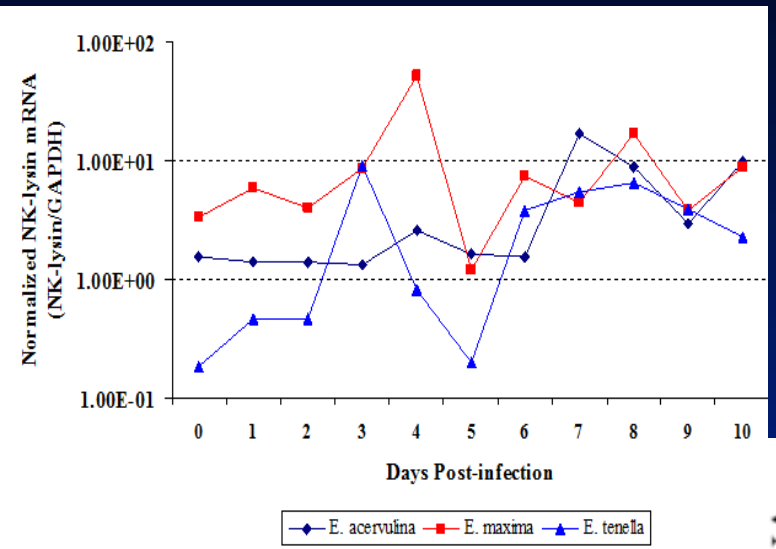


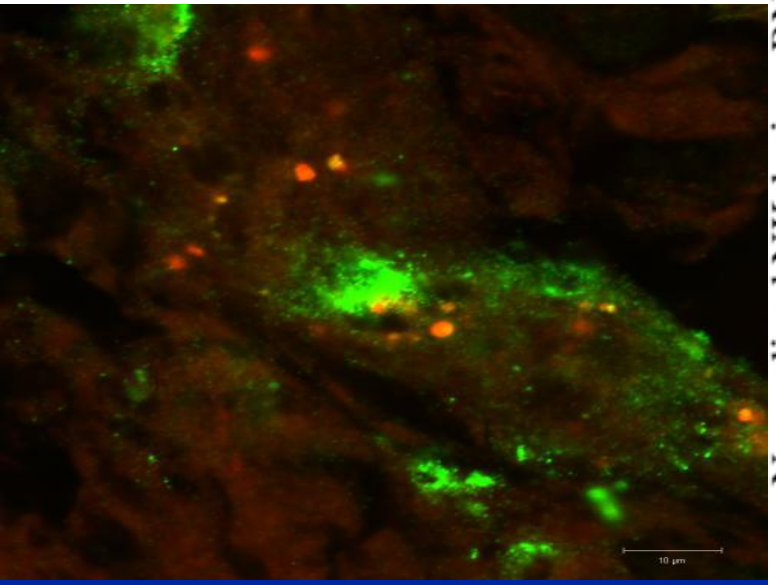


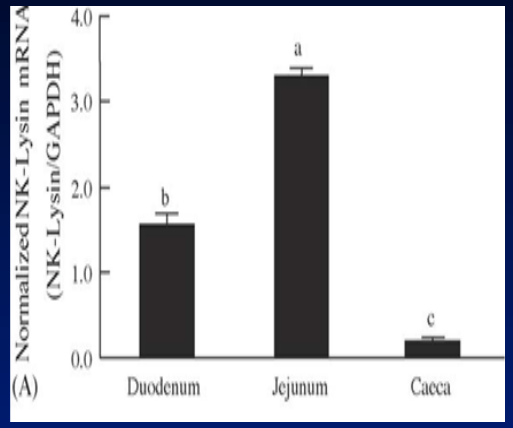
COS7 cells were transfected with pTriEx4-NK-lysin, rchNK-lysin purified on Ni2+-NTA His column.

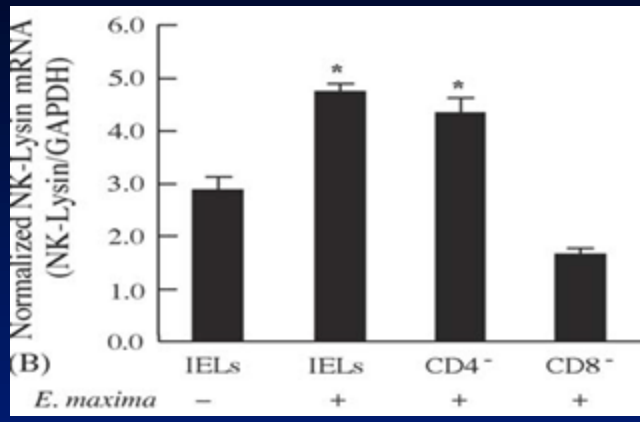


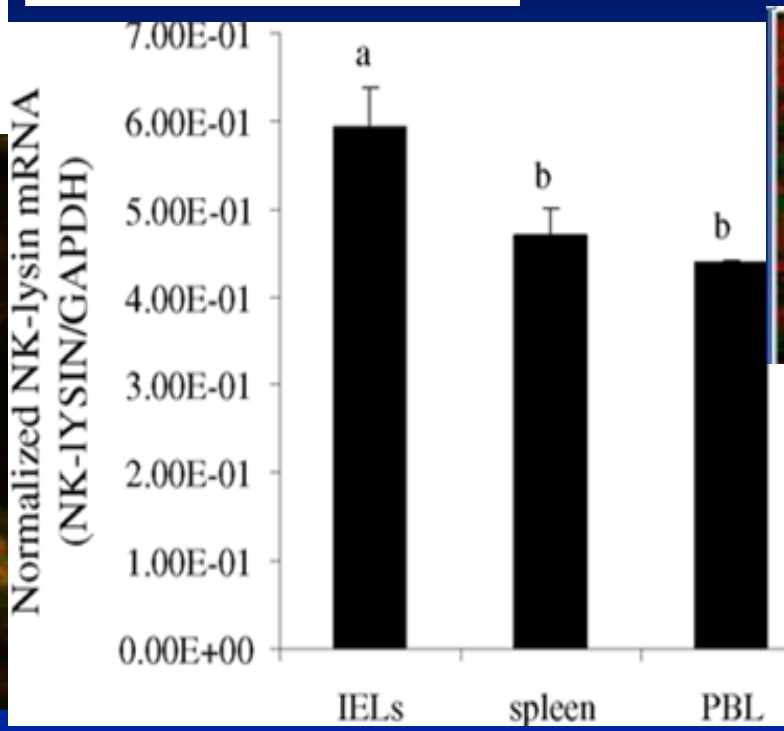
NK lysin secretion and tissue distribution in coccidiosis

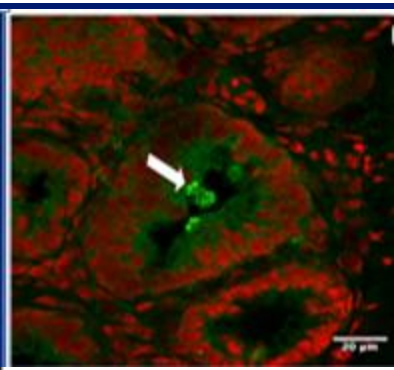






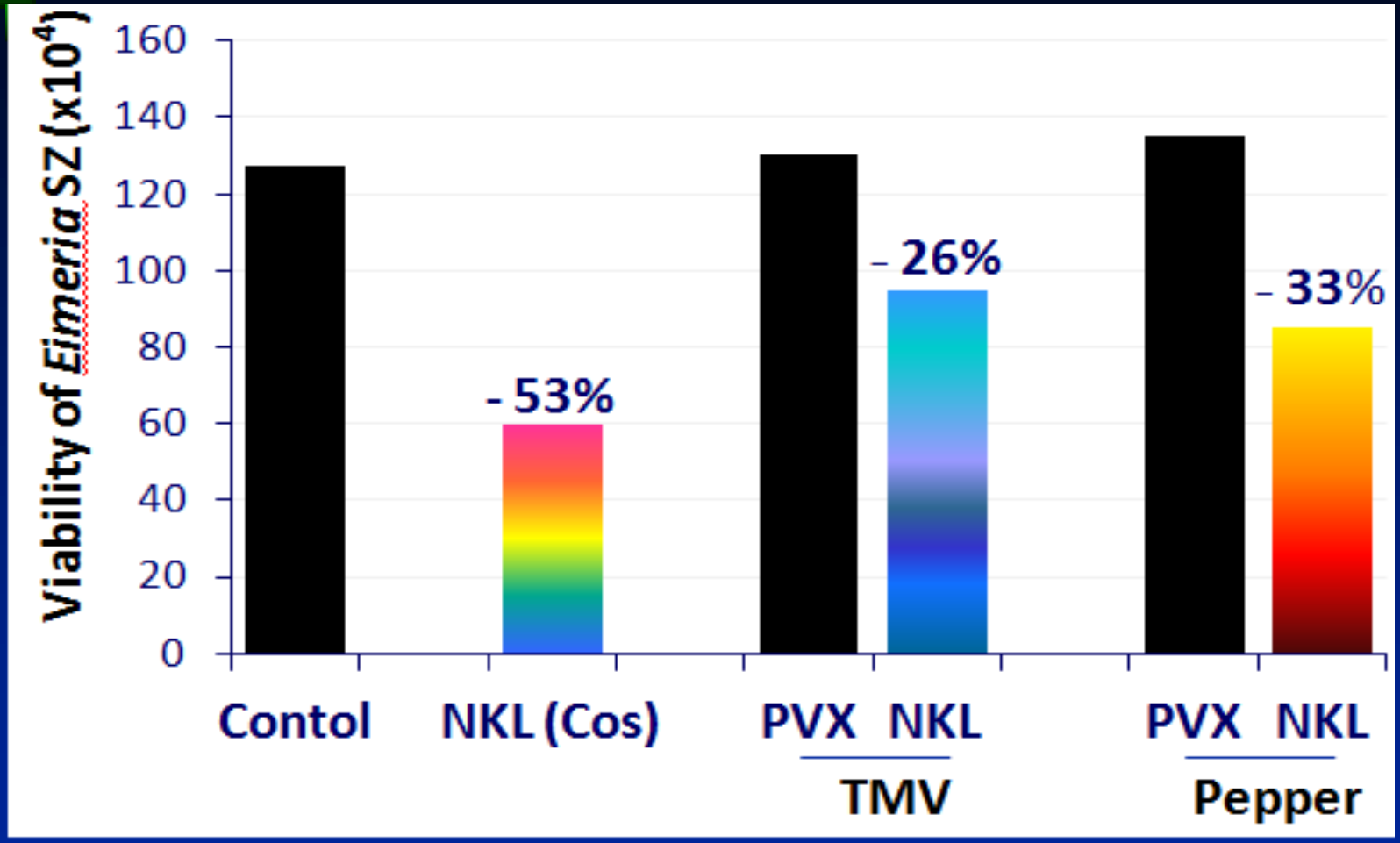


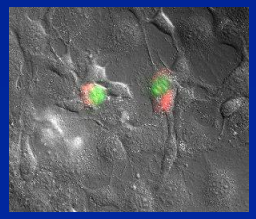




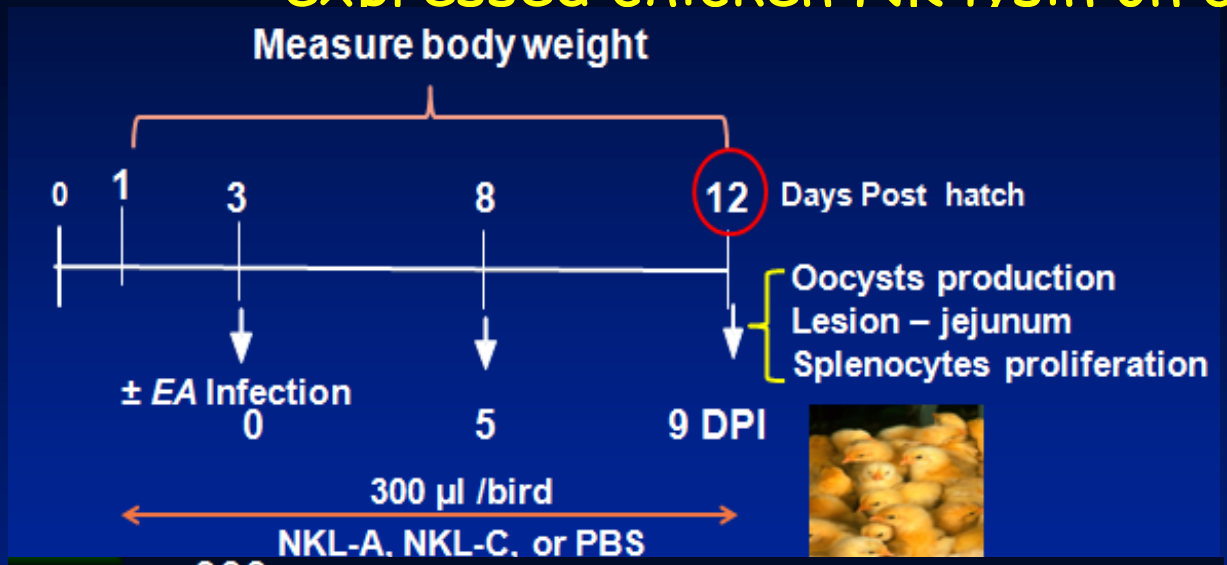
Anti-microbial activity of recombinant chicken NK lysin proteins (In vitro and in vivo studies)

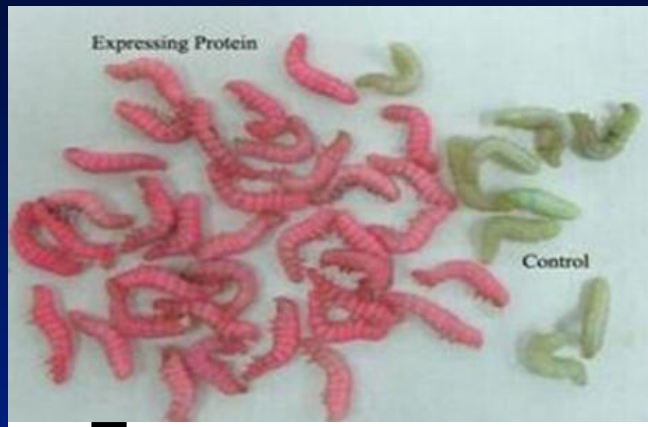
In vitro effects of NK lysin expressed in plant vectors

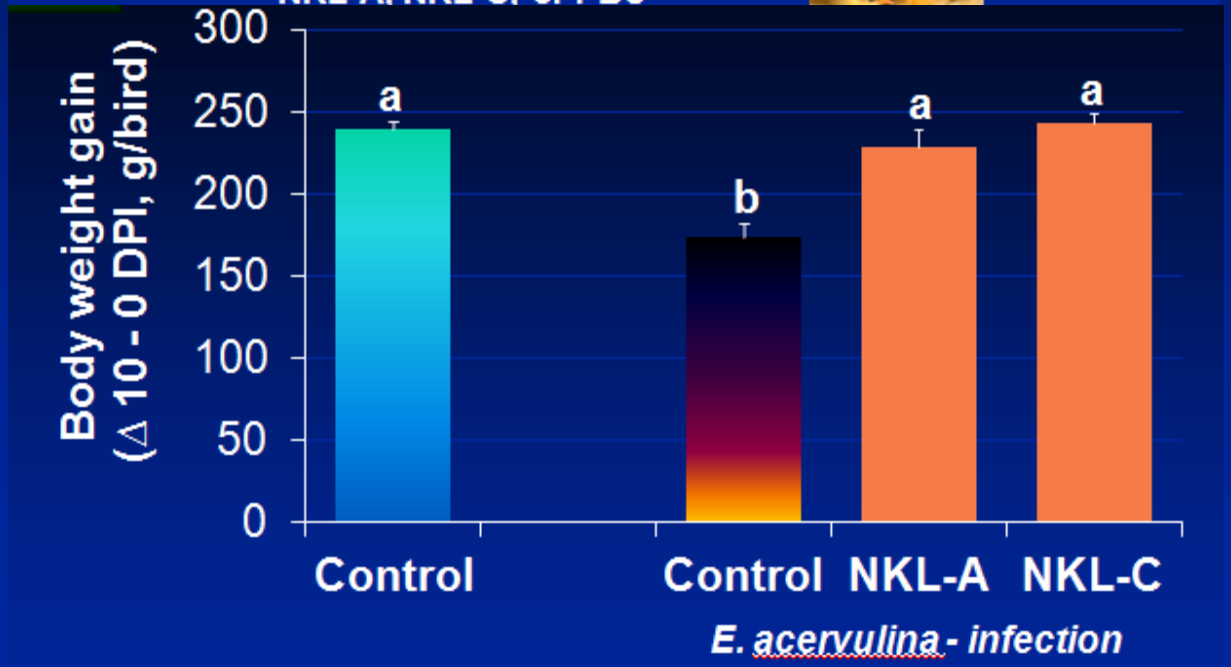


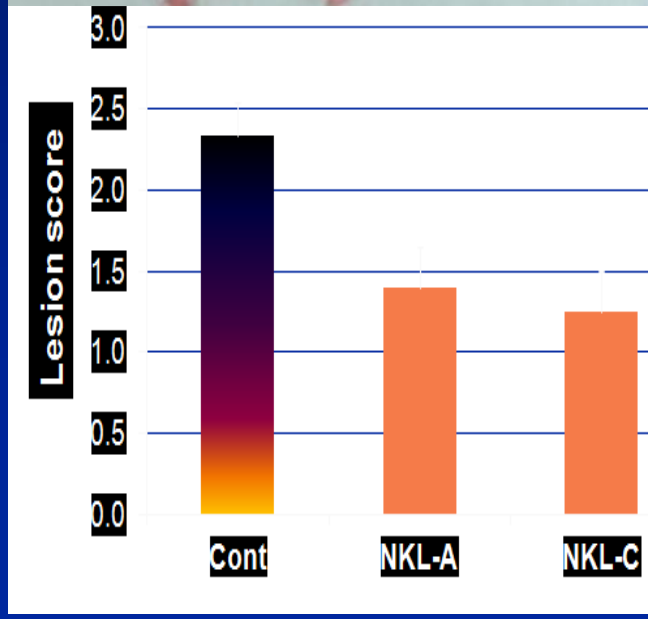


Effect of oral feeding of silk worm-expressed chicken NK lysin on coccidiosis

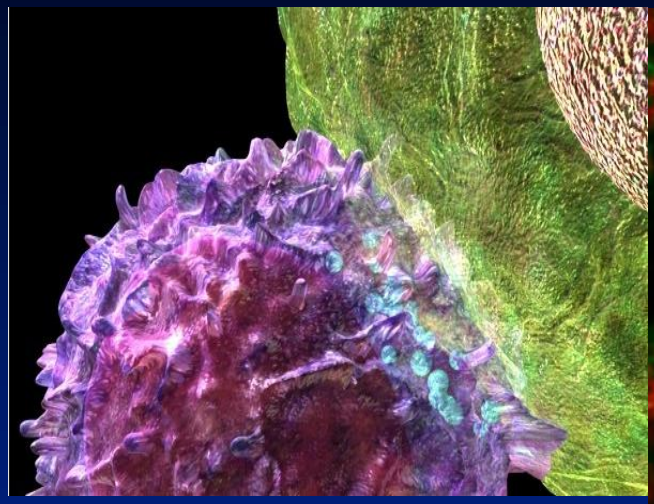


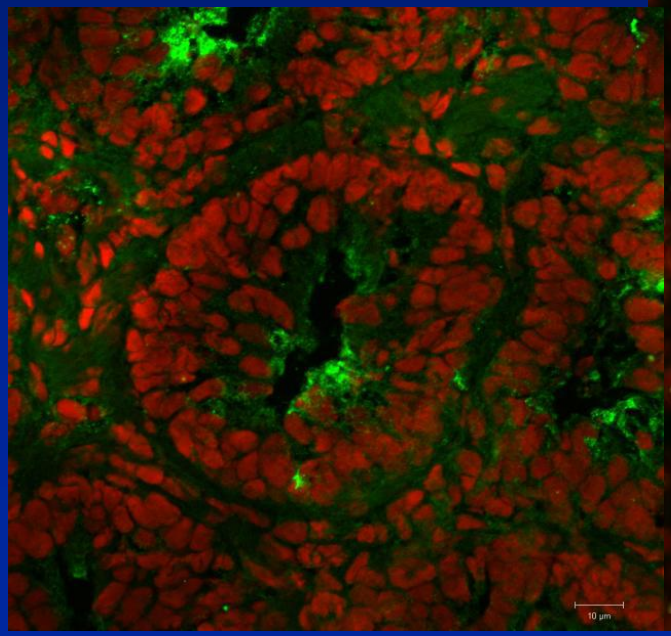


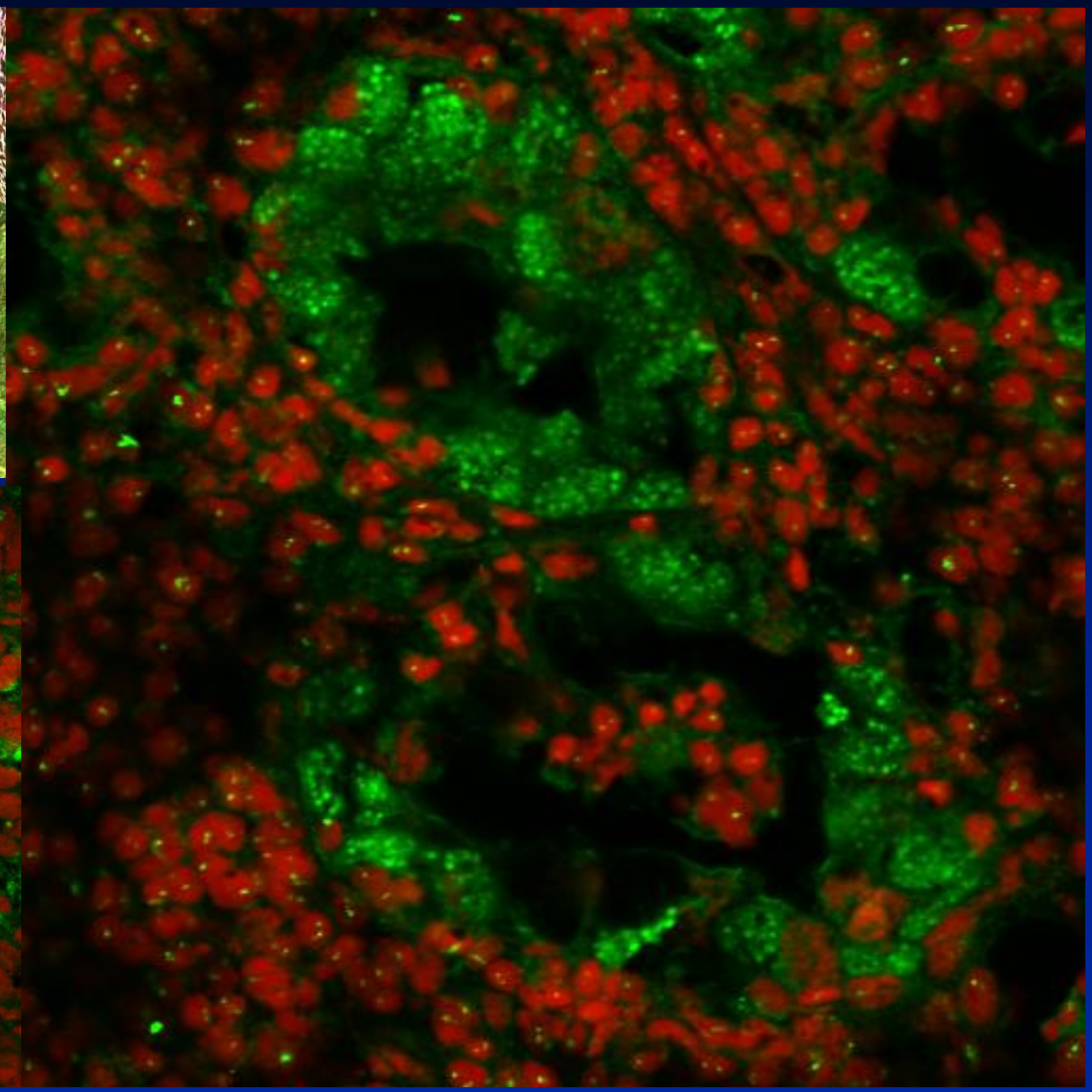




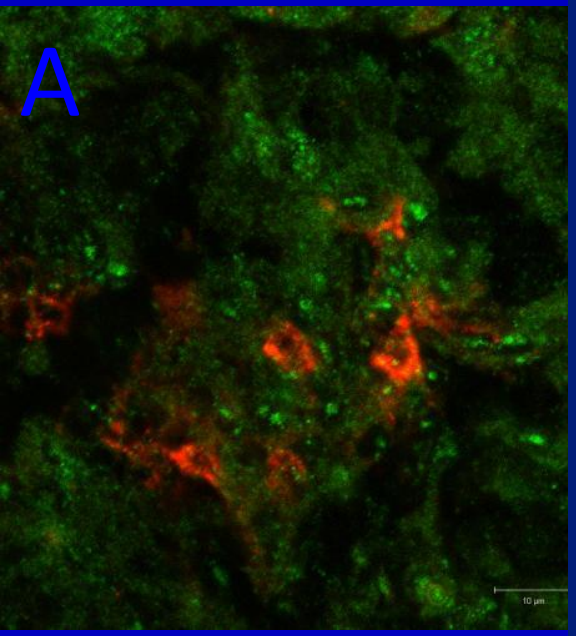
Local NK lysin secretion: Mode of action?



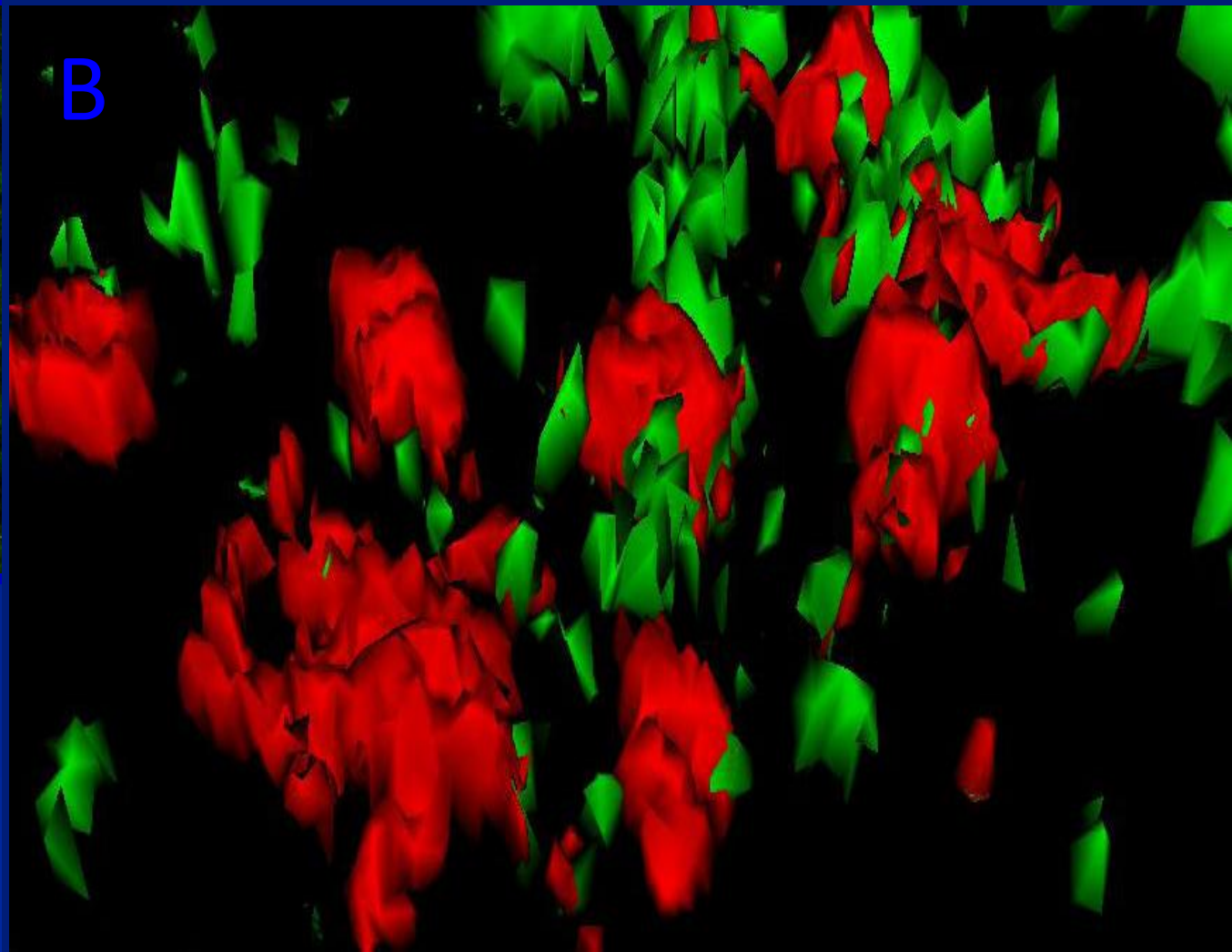




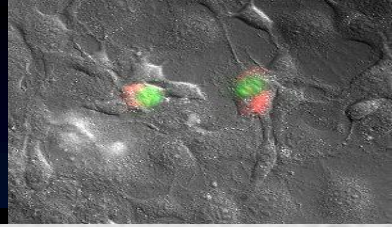
Two-color immunofluoresence showing NK cells secreting NK lysin:
Red are NK cells and green are secretory NK lysin proteins (A).
B shows surface-enhanced topography of NK cells secreting NK lysin.

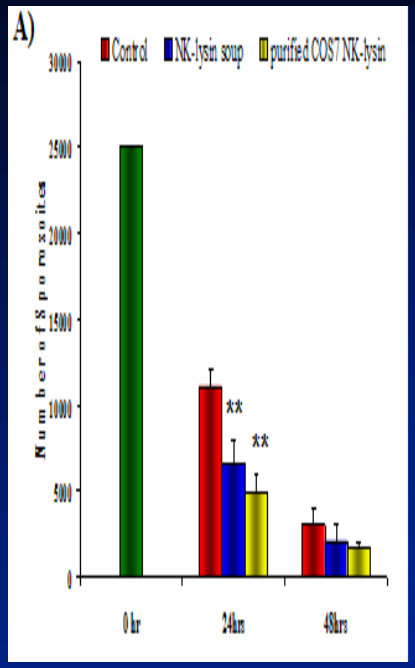


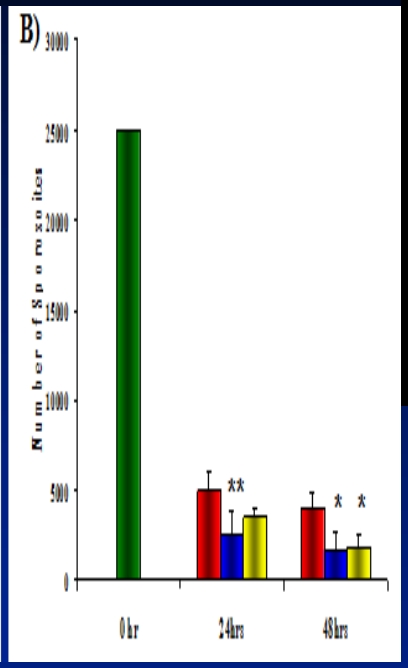
CD4+ and CD8+ cells expressed equal amounts of NK lysin, but IELs depleted of CD8+ cells exhibited reduced NK-lysin transcript levels. (Hong et al., 2008)

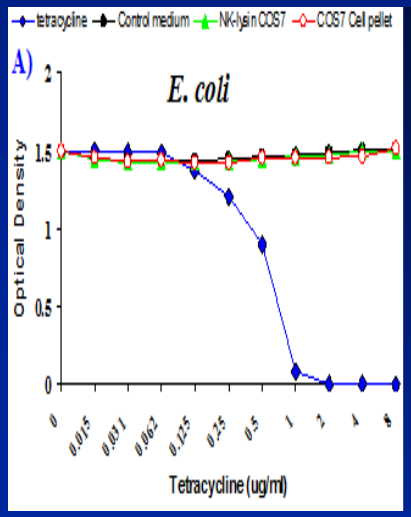


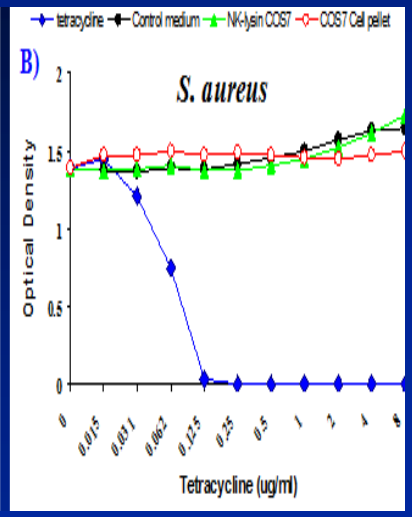
Cytotoxicity of rchNK lysin (Cos7)

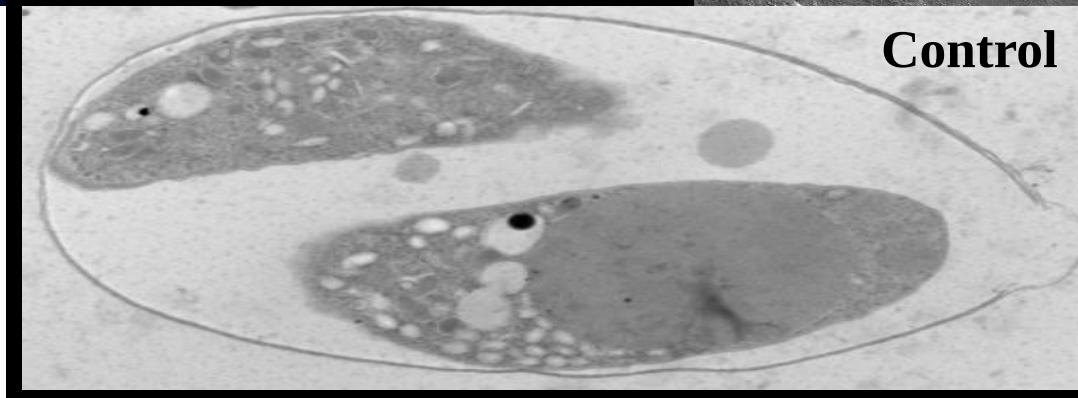


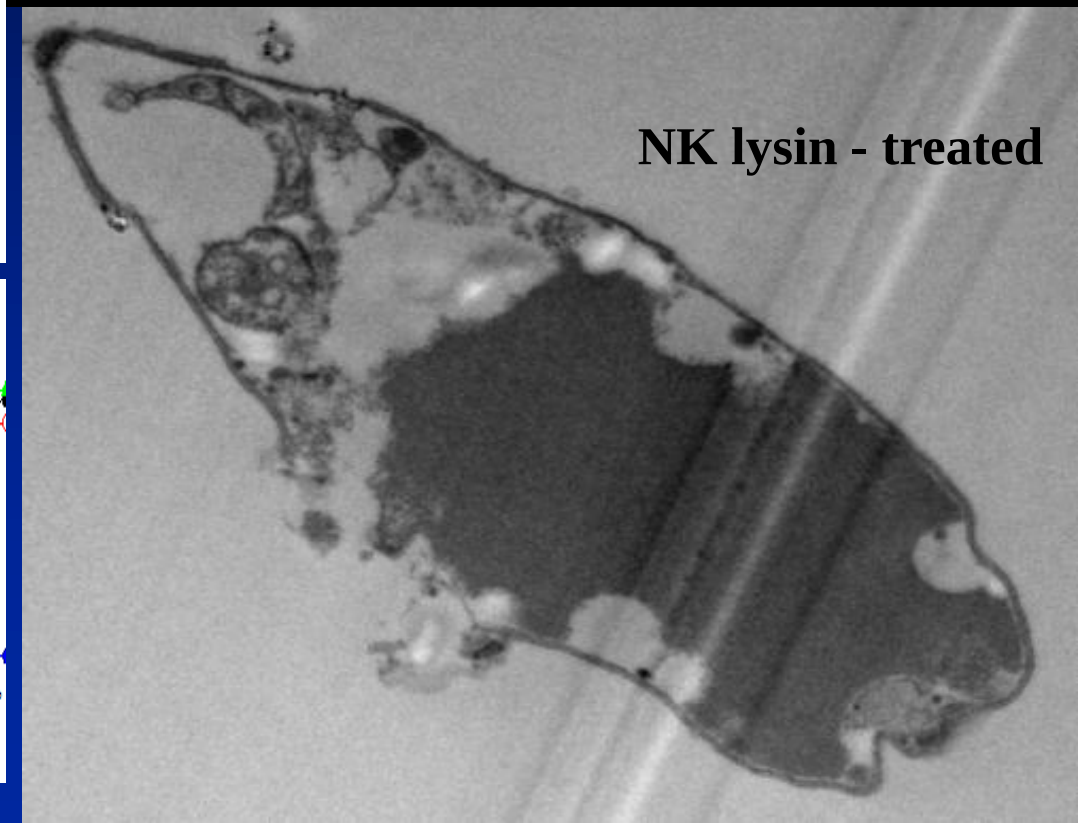












Effects of a single nucleotide polymorphism in the chicken NK-lysin gene on antimicrobial activity and cytotoxicity of cancer cells.

Lee et al., 2012 (www.pnas.org/cgi/doi/10.1073/pnas.1209161109)

- White leghorn & Cornish chickens
- Genomic DNA from blood
- Synthesis of NK-lysin SNP region

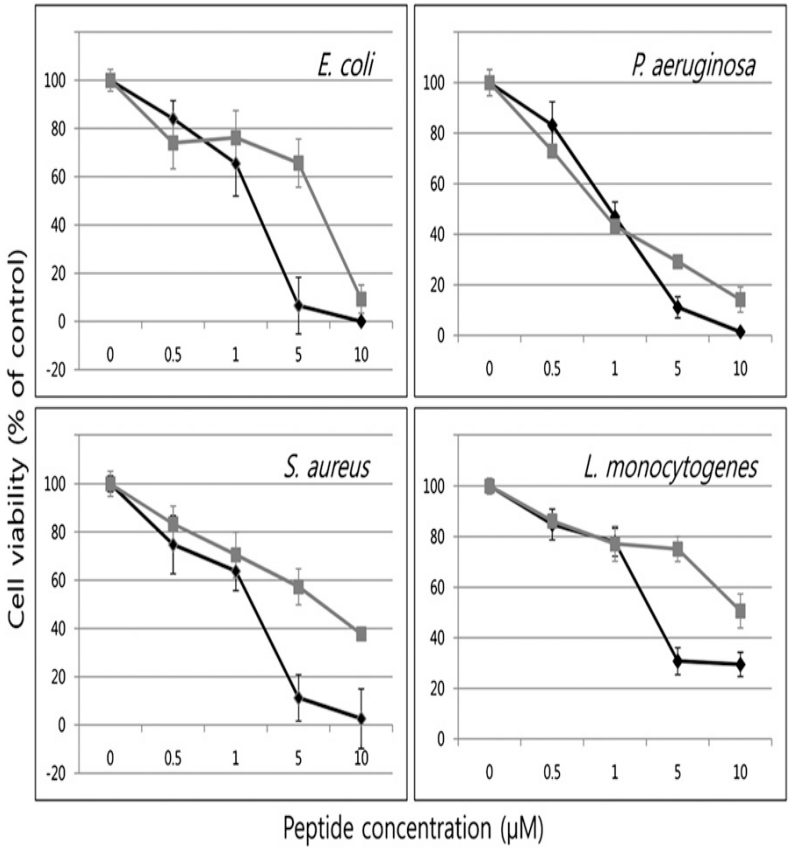
D A I N **N** A L N K
D A I N **D** A L N K

(N) to Asp (D) amino acid alteration produced by nonsynonymous SNP

Sequences and properties of NK-lysin peptides

Peptide	Sequence	Length (aa)	Mw	Net charge	Hydrophobic ratio
N29N	PDEDAIN**N**ALNKVCSTGRRQRSICKQLLKK	30	3399.9	3.9	33
N29D	PDEDAIN**D**ALNKVCSTGRRQRSICKQLLKK	30	3400.9	2.9	33

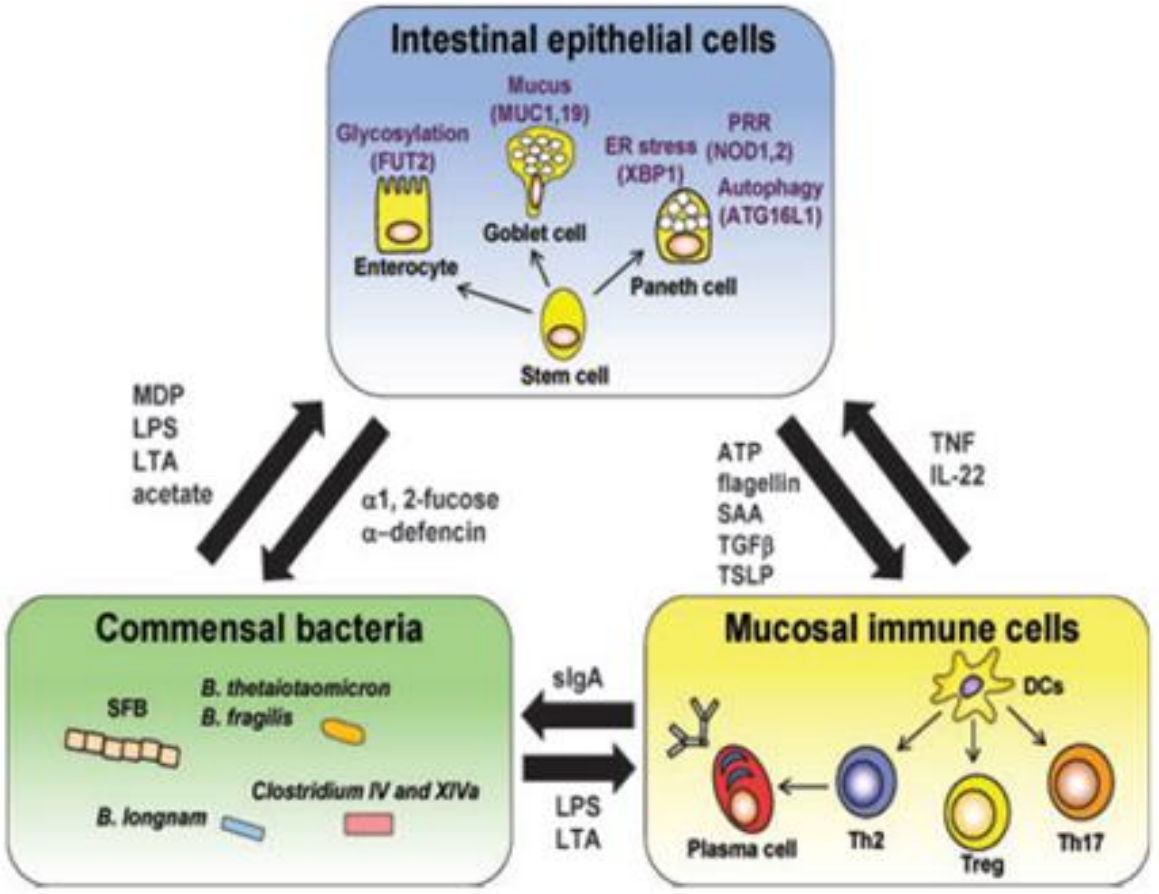
Mw, molecular weight. Substituted amino acid is boldface.



Antibacterial properties of N29N and N29D peptides

Animal Well-Being

"Maintaining intestinal homeostasis "



Goto & Kiyono, 2011.

Tomorrow's Poultry

The good news is we're *organic*, *free-ranged* and *antibiotic-free*.

The bad news is that we'll be eaten by people who eat *organic*, *free-range*, *antibiotic-free*…..

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